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Request for grant of a patent

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1. Your reference — 4-30760/P1

2. Patent application number *(The Patent Office will fill in this part)* — **9828709.7**

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

NOVARTIS AG
Schwarzwaldallee 215
4058 Basel

Patents ADP number *(if you know it)* — 7125487002

If the applicant is a corporate body, give the country/state of its incorporation — Switzerland

4. Title of the invention — Assay

5. Name of your agent *(if you have one)*

B. A. Yorke & Co.

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

Coomb House
7 St. John's Road
Isleworth, Middlesex TW7 6NH

Patents ADP number *(if you know it)* — 1800001

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Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from a earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer "Yes" if:*

a) any applicant named in part 3 is not an inventor , or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.
See note (d))

Yes

ASSAY

This invention relates to an assay for the identification of biologically active compounds, in particular to a reporter gene assay for the identification of compounds which have an effect on mRNA stability.

Recently, it has become increasingly apparent that the regulation of RNA half-life plays a critical role in the tight control of gene expression and that mRNA degradation is a highly controlled process. RNA instability allows for rapid up- or down-regulation of mRNA transcript levels upon changes in transcription rates. A number of critical cellular factors, e.g. transcription factors such as c-myc, or gene products which are involved in the host immune response such as cytokines, are required to be present only transiently to perform their normal functions. Transient stabilisation of the mRNAs which code for these factors permits accumulation and translation of these messages to express the desired cellular factors when required; whereas, under non-stabilised, normal conditions the rapid turnover rates of these mRNAs effectively limit and "switch off" expression of the cellular factors. However, abnormal regulation of mRNA stabilisation can lead to unwanted build up of cellular factors leading to undesirable cell transformation, e.g. tumour formation, or inappropriate and tissue damaging inflammatory responses.

Although the mechanisms which control mRNA stability are far from understood, sequence regions have been identified in a number of mRNAs, which appear to confer instability on the mRNAs which contain them. These sequence regions are referred to herein as "mRNA instability sequences". For example, typical mRNA instability sequences are the AREs (AU rich elements), which are found in the 3'UTR (3' untranslated region) of certain genes including a number of immediate early genes and genes coding for inflammatory cytokines, e.g. IL-ß and TNFα.

As described in our copending British patent application of even date herewith, entitled "ORGANIC COMPOUNDS", we have discovered compounds which promote instability of mRNAs which contain mRNA instability sequences. Such compounds may be used to induce degradation of mRNAs, thus preventing or reversing inappropriate mRNA accumulation and thereby decreasing or preventing unwanted protein, e.g. cytokine, expression. Thus such compounds are potentially useful pharmaceutically for prophylaxis or treatment of diseases or medical conditions which involve inappropriate mRNA stabilisation and accumulation and resultant undesirable protein expression.

The present invention relates to a reporter gene assay for identifying compounds which affect the stability of mRNAs which contain mRNA instability sequences.

ambit of the skilled worker in the art, and does not form a substantive part of the invention. Thus, for instance, for expression in mammalian cells a viral promoter such as an SV40, CMV or HSV-1 promoter may be used. On the other hand appropriate choice of mRNA instability sequence is of importance to the successful functioning of the reporter gene assay and forms part of the invention.

Thus in a further aspect the invention provides a reporter gene DNA expression system comprising a gene coding for expression of a protein having a detectable signal, wherein the gene comprises DNA coding for the amino acid sequence of the protein together with associated 5' and 3' UTR sequences comprising appropriate expression control elements and DNA corresponding to at least one copy of a mRNA instability sequence.

mRNA instability sequences have been identified in the UTRs, in particular the 3' UTRs, of a large number of transiently expressed genes including genes for cytokines, chemokines, nuclear transcription factors, protooncogenes, immediate early genes, cell cycle controlling genes, oxygenases, genes involved in and controlling of apoptosis. The natural RNA sequences which comprise the mRNA instability sequences are alternatively referred to as adenylate/uridylate (AU)-rich elements, or AREs. Transiently expressed genes which contain mRNA instability sequences include, for example, the genes coding for GM-CSF, c-*fos*, c-*myc*, c-*jun*, *krox*-20, *nur*-77, *zif268*, bcl-2, β-IFN, uPA, IL-1, IL-3, TNF-α, MCP1, syn1, β_2-AR, E-selectin, VCAM-1, ICAM-1, MMP-1, MMP-2, collagenases, P-glycoproteins (MDR), MRPs, Pγh1 (pf mdr), COXII, and MIP-2α.

The following publications include extensive disussion of mRNA instability sequences and AREs, the sequences motifs which they contain and (minimum) sequence requirements for mRNA destabilisation, as well as identifying a number of mRNA instability sequences and the genes which contain them:

Shaw & Kamen, Cell, Vol. 46, 659-667, August 29 1986 (GM-CSF);

Shyu et al., Genes & Development, 5:221-231 (1991) (c-*fos*);

Sachs, Cell, Vol. 74, 413-421, August 13 1993 (Review. "Messenger RNA Degradation in Eukaryotes");

Chen et al., Mol. Cell. Biol., Jan 1994, p 416-426 (c-*fos*);

Akashi et al., Blood, Vol. 83, No. 11, (June 1), 1994: pp 3182-3187 (GM-CSF etc.);

Nanbu et al., Mol. Cell. Biol., July 1994, p. 4920-4920 (Upa);

Stoecklin et al., J. Biol. Chem., Vol. 269, No. 46, November 18 1994, pp 28591-28597 (IL-3);

Lagnado et al., Mol. Cell. Biol., Dec. 1994, p. 7984-7995 (general);

Zhang et al., Mol. Cell. Biol., Apr. 1995, p. 2231-2244 (yeast);

or osteoarthritis are preferably detected using a reporter gene expression system comprising an IL-1 mRNA instability sequence.

Thus by way of illustration of the invention a preferred mRNA instability sequence for use in the identification of compounds which destabilise IL-β mRNA is derived from the 3' UTR of IL-1β mRNA, e.g. the sequence given hereafter as SEQ ID NO: 5. More preferably the IL-1β mRNA instability sequence may comprise a fragment of the 3' UTR of IL-β mRNA. For example, a particularly preferred IL-1β mRNA instability sequence comprises the 30 nucleotide sequence derived from the 3' UTR of IL-1β mRNA given hereafter as SEQ ID NO: 6.

Preferably the mRNA instability sequence is located in the 3' UTR of the reporter gene. Thus for example, the DNA sequence corresponding to the mRNA instability sequence is inserted as or as part of an appropriate DNA segment into a suitable restriction site in the 3' UTR of the native reporter gene.

The DNA expression system is preferably a cell based expression system, conveniently in the form of a suitably transformed cell line, preferably a stably transformed cell line. The host cell is typically an eucaryotic host cell, in particular an animal host cell, especially a mammalian host cell.

Preferably the host cell is of the same general cell type as the cells which express the protein which is coded for by the mRNA which it is desired to destabilise. Thus for instance, if the assay of the invention is to be used for the identification of compounds which destabilise the mRNA coding for a cytokine, the host cell used is preferably a cell or cell line which is of the same or similar cell type to the cells which normally produce the cytokine in question. For example, monocyte or monocyte-like cell lines may be used as host cells for assaying for compounds which destabilise cytokine, e.g. IL-1β, mRNA. Preferred cell lines for oncogene and other cancer related gene mRNA instability assays are, e.g. Colon 205, KB 31, KB 8511, DU-145, HCT116, MCF7, MCF7/ADR, MDA-MB-231, MDA-MB-435 and MDA-MB-435/TO. Particularly preferred cell lines for use as the host cells in assays of the invention for identification of compounds which destabilise cytokine, e.g. IL-1β, mRNA are the THP-1 cell line (for instance as described by Auwerx J. (1991), Experientia, 47: 22-30) and similar monocytic, e.g. human leukaemia, cell lines.

Preferably also, the mRNA instability sequence and the host cell are derived from the native mRNA which it is desired to destabilise and the native cell type in which that mRNA is produced respectively. Thus for instance, for identification of compounds which destabilise cytokine mRNA, the mRNA instability sequence is preferably derived from the mRNA which codes for the cytokine in question and the host cell is preferably of the same cell type as the native cell type in which the

system, i.e. contains sequence coding for expression of the detectable protein but which does not contain sequence corresponding to a mRNA instability sequence. Preferably the control DNA expression system is identical to the reporter gene expression system except that the DNA corresponding to the mRNA instability sequence has been removed, deleted or otherwise disabled as a mRNA instability sequence. Preferably the control DNA expression system is also in the form of a transformed cell line, typically a stably transformed cell line derived from the same host cell line, e.g. a THP-1 cell line, as the reporter gene transformed cell line.

Accordingly in a preferred embodiment the invention provides an assay system for the identification of compounds which destabilise mRNA comprising

a reporter gene DNA expression system as defined above, and

a control DNA expression system which comprises a gene coding for expression of the protein having the detectable signal, wherein the gene comprises DNA coding for the amino acid sequence of the protein together with associated 5' and 3' UTR sequences comprising appropriate expression control elements but lacking a function mRNA instability sequence.

Preferably both the reporter gene DNA expression system and the control DNA expression system are in the form of stably transfected cell lines.

Alternatively the reporter gene expression system may be tested in the presence and absence of the test compound, testing in the absence of the test compound being used as the control.

The invention is further described by way of illustration of the invention only in the following Examples which relate to a particular assay of the invention and refer to the accompanying Figures:

Figure 1 which shows the DNA sequence of IL-1β 3' UTR;

Figure 2 which shows the 30 bp fragment used as a mRNA instability sequence in Example 1;

Figure 3 which shows plasmid diagrams for pGL2_Neo30 and pGL2-Control;

Figure 4 which shows graphs of luciferase activity over the time of differentiation for clone No. 53 (A) and clone No. 63 (B);

Figure 5 shows graphs of luciferase half lives, 4 and 8 hous after addition of compounds for clones 53 and 63 treated with radicicol analog A (SDZ 216-732), actinomycin D (act D.) and cyclohexamide (CHX);

Figure 6 shows graphs of luciferase activity from clones 53 (solid bars) and 63 (open bars) treated with various concentrations of radicicol analog A (SDZ 216-732);

Figure 7 shows graphs of luciferase activity for undifferentiated (undiff) and differentiated (diff) clone 53 (solid bars) and clone 63(open bars) treated with radicicol analog A, and

Modification of pGL2-Control (Promega) by introducing a neomycin resistant marker gene (expressing aminoglycoside 3' phosphotransferase) and by adding 30bp of IL1β 3UTR sequence results in the luciferase expression vector pGL2_Neo30 (Fig. 3). Fig. 2 shows the IL-1β 3'UTR sequence containing three tandem AUUUA motifs used for ligation into the PflMI site of pGL2-Control.

The resulting vector (pGL2_neo30) is cotransfected with pGL2-Control into THP-1 cells by electroporation. 10^7 cells/ml in 1.3mM KH_2P0_4, 7.36m.M Na_2HP0_4, 2.44mM KCl, 124mm NaCl, 5mM glucose, 9.6μM $MgCl_2$ and 16μM $CaCI_2$ pH 7.2 are transfected with 20μg of DNA in a Bio-Rad Gene Pulser (250V, 690μF and indefinite resistance) using a 0.4cm cuvette. Cells are subsequently cultured in RPMI medium containing 10%FBS, 2mM L-Gln (L-glutamine), 50μM 2-mercaptoethanol and 600μg/ml of G418 (geneticin). After transfection of pGL2_Neo30 and pGL2-Control into THP-1 cells, stable cell lines are obtained by selection for G418 resistance and assayed for luciferase activity. One cell line of each transfection is chosen for further analysis; the pGL2_Neo30 cell line is referred to as clone No. 63 and the pGL2-Control cell line as clone No. 53. No endogenous luciferase activity could be detected in normal THP1 cells.

The tissue culture and luciferase activity measurements are carried out as described below.

Tissue culture:

The transfected human monocytic leukaemia cell lines, clones No. 53 and 63 are grown in RPMI medium supplemented with 110 U/ml penicillin, 100μg/ml streptomycin, 2 mM L-Gln and 2 g/1 $NaHCO_3$. Heat-treated FBS (5%) is added before use. The cells are grown to a density of 5x 10^5/ml and induced to differentiate with 100 U/ml (final concentration)γIFN. Three hours later, 10 μl of LPS (5μg/ml final concentration) is added. This time point is designated time 0. Compounds are added at various times after LPS addition as indicated.

Luciferase activity measurement:

In order to adapt the system to the use of 96 well plates, cells are grown in Packard flat bottom white polystyrene microplates (Cat. No.6005180) in RPM1 medium lacking phenol red (AMIMED). Cells are plated at $5x10^4$/well. After treatment of the cells, luciferase is measured using the Packard Luc Lite system (Cat. No.601691 1) according to the manufacturer's instructions in a final volume of 205μl. Briefly, to a cell suspension of 5 x 10^6 cells/ml, γIFN (1000U/ml Boehringer Mannheim No. 1050494) to a final concentration of 100 U/ml and 0.25% (v/v) Luc Lite Enhancer is

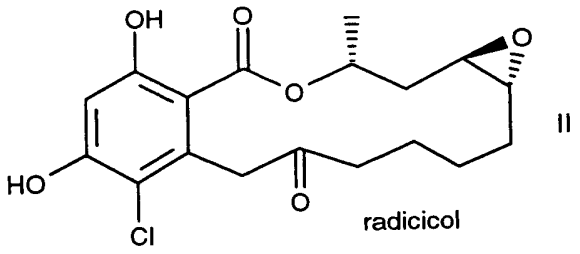
The THP-1 cell lines, clone No. 63 (containing PGL2_Neo30) and clone No. 53 (containing pGL2-Control) are grown, differentiated and stimulated with γIFN and LPS identical to normal THP-1 cells. Radicicol analog A is added 16 hours after the addition of LPS and cell extracts are then taken 8 hours later or as indicated. Luciferase activity is inhibited by 1μM radicicol analog A on average by 50% +/-17%, in some cases inhibition was as great as 93%, whereas up to $5x10^{-6}$M of radicicol analog A has no effects on the control clone No. 53, Fig. 6 (solid bars indicate clone No. 53, open bars clone No. 63).

Interestingly, undifferentiated (undiff) clone No. 63 (open bars) when treated with radicicol analog A showed only a limited reduction of luciferase activity (Fig. 7, solid bars indicate clone No. 53), which is either due to the lower expression of luciferase or is indicative of the involvement of a differentially expressed or modified component in the mRNA degradation process mediated by AU-rich elements. Indeed, gel retardation experiments using 241 bp of the AU-rich 3' UTR of IL-1β as a riboprobe showed the binding of additional proteins with γIFN induced differentiation or modification (not shown).

Concentration dependent inhibition of luciferase activity is shown in Fig. 8. Concentrations of radicicol analog A higher than $5x10^{-6}$M also inhibited the control clone due to cytotoxicity or inhibitory activity on transcription.

Example 4: Application of assay to a number of selected substances

A number of selected substances are tested for their activity in the assay of the invention substantially as described in the previous Examples. The results obtained are given in the Table below. Radicicol (see formula II below) and radicicol analog A show a clear effect on mRNA stability; other compounds tested did not show activity in the assay used.



radicicol II

CLAIMS

1. A method for the identification of a compound which affects mRNA stability, in which a DNA expression system which in the absence of the test compound is capable of expressing a protein having a detectable signal, and wherein the mRNA which codes for the protein and which is transcribed from the expression system comprises at least one copy of a mRNA instability sequence is contacted with a test compound and the detectable signal is measured in the presence of the test compound and compared with a control.

2. A method according to claim 1, for the identification of a compound which induces mRNA degradation, comprising contacting the compound with a DNA expression system which in the absence of the compound is capable of expressing a protein having a detectable signal, wherein the mRNA which codes for the protein and which is transcribed from the expression system comprises at least one copy of a mRNA instability sequence, measuring the detectable signal in the presence of the test compound and comparing the result obtained with a control.

3. A method for the comparison of compounds which induce mRNA degradation, comprising separately contacting the compounds with a DNA expression system which in the absence of the compounds is capable of expressing a protein having a detectable signal, wherein the mRNA which codes for the protein and which is transcribed from the expression system comprises at least one copy of a mRNA instability sequence, measuring the detectable signal in the presence of each test compound and comparing the signals obtained.

4. A reporter gene DNA expression system comprising a gene coding for expression of a protein having a detectable signal, wherein the gene comprises DNA coding for the amino acid sequence of the protein together with associated 5' and 3' UTR sequences comprising appropriate expression control elements and DNA corresponding to at least one copy of a mRNA instability sequence.

5. A stably transfected cell line comprising a reporter gene DNA expression system according to claim 4.

ABSTRACT

ASSAY

A method is provided for the identification of a compound which affects mRNA stability, in particular induces mRNAdegradation, in which a DNA expression system which in the absence of test compound is capable of expressing a protein having a detectable signal, wherein the mRNA which codes for the protein and which is transcribed from the expression system comprises at least one copy of a mRNA instability sequence, is contacted with a test compound and the detectable signal is measured in the presence of the test compound and compared with a control. The method may be use to identify compounds which induce degradation of mRNA, e.g. cytokine (e.g. IL-β) mRNA, which when inappropriately stabilised can give rise to diseases or medical conditions, e.g. cytokine induced inflammatory disease.

*GGACCAAAGG CGGCCAGGAT ATAACTGACT TCACCATGCA

ATTTGTGTCT TCCTAAAGAG AGCTGTACCC AGAGAGTCCT

GTGCTGAATG TGGACTCAAT CCCTAGGGCT GGCAGAAAGG

GAACAGAAAG GTTTTTGAGT ACGGCTATAG CCTGGACTTT

CCTGTTGTCT ACACCAATGC CCAACTGCCT GCCTTAGGGT

AGTGCTAAGA GGATCTCCTG TCCATCAGCC AGGACAGTCA

GCTCTCTCCT TTCAGGGCCA ATCCCAGCCC TTTTGTTGAG

CCAGGCCTCT CTCACCTCTC CTACTCACTT AAAGCCCGCC

TGACAGAAAC CAGGCCACAT TTTGGTTCTA AGAAACCCTC

CTCTGTCATT CGCTCCCACA TTCTGATGAG CAACC GCTTC

CCT ATTTATTTATTTA TTTG TTTGT TTGTT TTGATTCATT

GGTCTA ATTTA TTCAAAGGG GGCAAGAAGT AGCAGTGTCT

GTAAAAGAGC CTAGTTTTTA ATAGCTATGG AATCAATTCA

ATTTGGACTG GTGTGCTCTC TTTAAATCAA GTCCTTTAAT

TAAGACTGAA AATATATAAG CTCAGATT ATTTAAATGGGA

AT ATTTA TAA ATGAGCAAAT ATCATACTGT TCAATGGTTC

TCA AATAAA C TTCAGT

Figure 1

ATGGCTTCCCTATTTATTTATTTATTTGTTTGTCCAACCT

GGATACCGAAGGGATAAATAAATAAATAAACAAACAGGTT

Figure 2

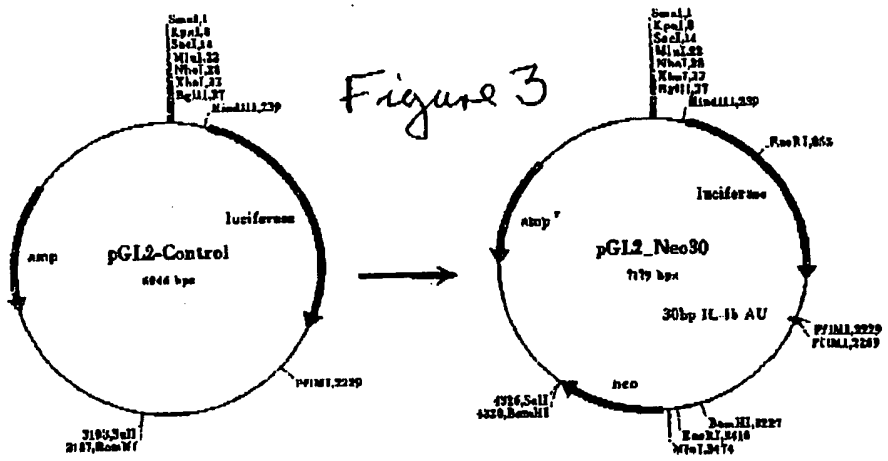


Figure 3

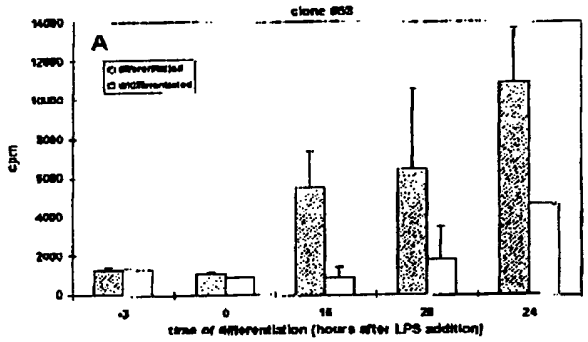
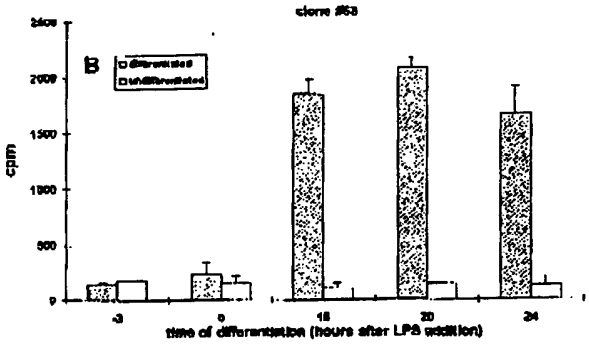


Figure 4



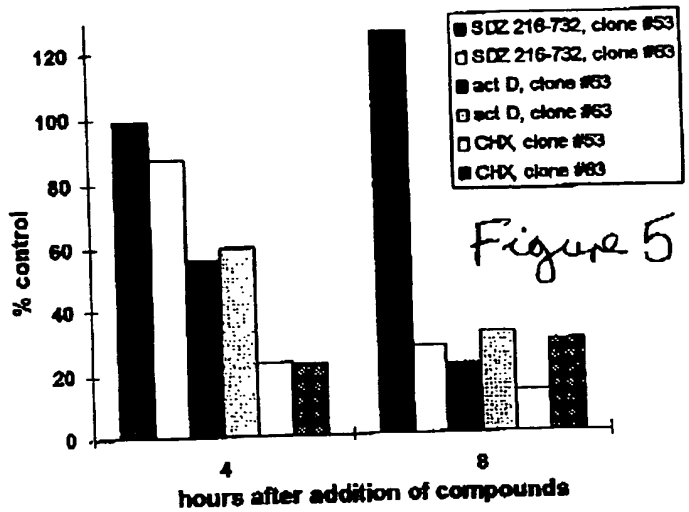


Figure 5

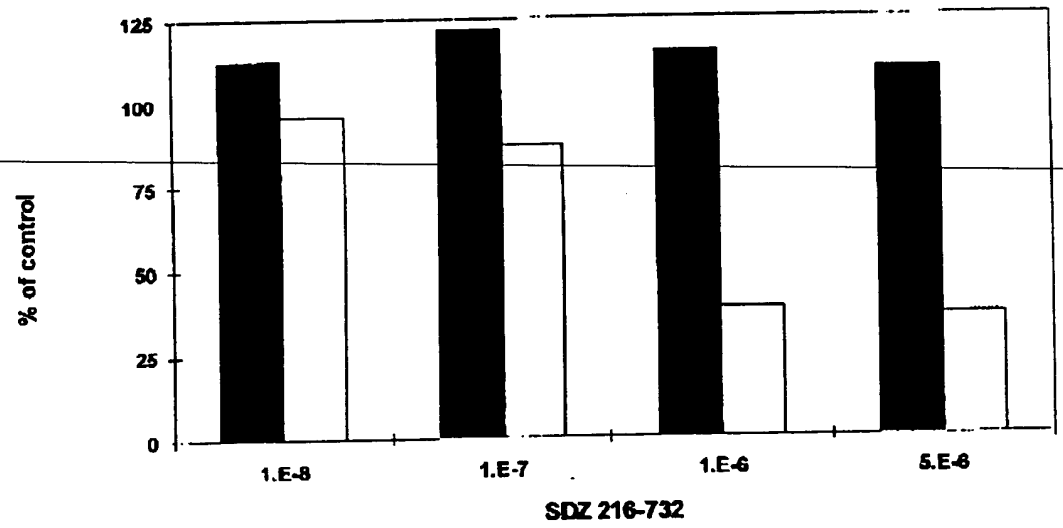


Figure 6.

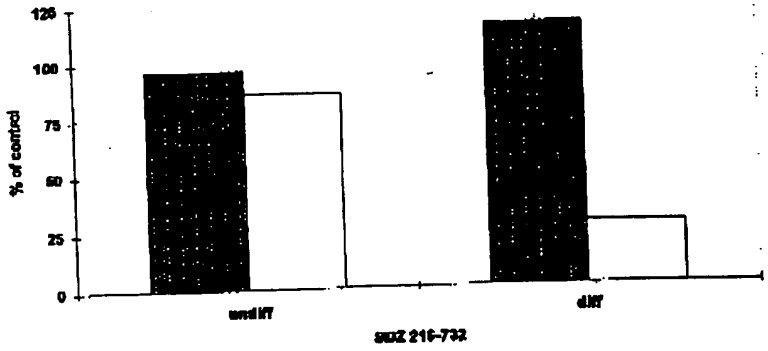


Figure 7

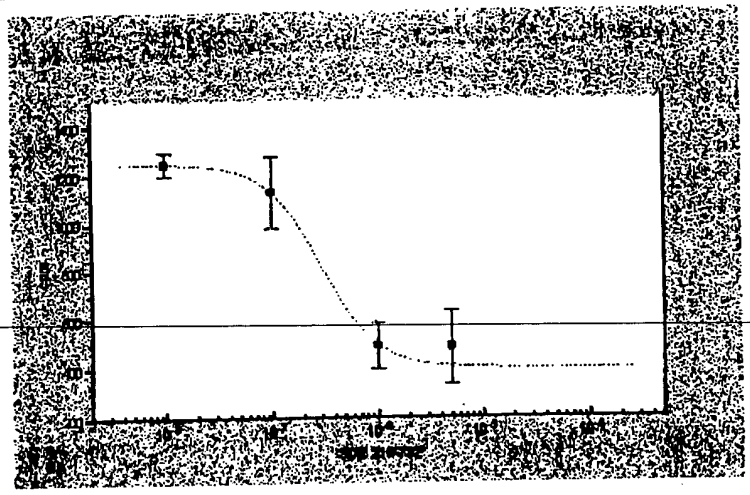


Figure 8